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Sister Chromatid Exchange and Micronucleus Frequency in Human Lymphocytes of 1,650 Subjects in an Italian Population: II. Contribution of Sex, Age, and Lifestyle

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Sister chromatid exchange (SCE) and micronuclei (MN) analysis was carried out on 1,650 healthy individuals living in Pisa and in two nearby small cities, Cascina and Navacchio (Ca-Na). The effect of smoking on SCEs was linearly correlated with the number of cigarettes per day, and an increase of 7.3% SCEs was detectable for as few cigarettes as 1–10/day. Ex-smokers showed intermediate mean values of SCEs (8.09 ± 1.88) in comparison with never smokers (7.54 ± 1.61) and current smokers (8.45 ± 1.94). Mean values of SCEs of ex-smokers decreased linearly with time of smoking cessation, reaching the mean values of never smokers within 8 years. The extent of SCE decrease was inversely proportional to the number of cigarettes previously smoked. No interaction between smoking habits and coffee or alcohol drinking on SCEs was observed. A borderline ($P = 0.053$) increase in mean SCE values in coffee drinkers (more than 3 cups/day) was found. The age effect on SCEs was remarkable in Ca-Na, but not in Pisa donors. Job type was not associated with significant modification of mean values of SCEs. Multiple logistic regression analysis revealed a statistically significant association between the proportion of high frequency cells (HCF) outliers and coffee consumption. Age and sex appeared to be by far the most important variables associated with modifications in MN frequency, which increased by 0.04‰ and 0.02‰ per year in males and females, respectively. Children and young donors (age ≤ 40 years) showed lower MN frequency regardless of sex, whereas sex appeared to determine a significantly higher increase of MN only in females older than 40 years. In contrast, in males the MN rate by age tended to level off after the age of 30–50. MN frequencies of Pisa blue- and white-collar workers were statistically significantly higher than in students (+0.71 and +0.55‰, respectively). Smoking did not determine any increase of MN frequency. A total lack of correlation ($P = 0.913$) between MN and SCEs was observed. Environ. Mol. Mutagen. 31:228–242, 1998

Key words: sister chromatid exchanges; HFCs; human monitoring; biological and lifestyle factors

INTRODUCTION

The influence of some lifestyle and biological factors on mean values of sister chromatid exchanges (SCEs) and micronuclei (MN) frequencies in human lymphocytes has been repeatedly studied. In particular, in spite of some discrepancies [Bender et al., 1989], age and sex have been shown to significantly modify SCE and MN frequencies [Morgan and Crossen, 1977; de Arce, 1981; Hedner et al., 1982; Soper et al., 1984; Anderson et al., 1986; Bender et al., 1988; Bonassi et al., 1995]. Females and older subjects exhibited higher mean values of SCEs and MN in comparison to males and young people, regardless of smoking habits. Mean values of SCEs have also been found lower in children and are known to increase with age [Das et al., 1985; Wulf et al., 1986; Fenech and Morley, 1985; Brown et al., 1983; Ford and Russell, 1985; Nowinski et al., 1990; Richard et al., 1993]. However, some negative studies have been published suggesting that the evidence of an increased level of MN in female lymphocytes is inconclusive [Bonassi et al., 1995].

Smoking habit is associated with increased mean SCE values [de Arce, 1981; Soper et al., 1984; Sarto et al., 1985; Hirsch et al., 1992; Ho Park et al., 1992; Lazutka

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et al., 1994] in a dose-dependent manner whether the number of pack/day [Soper et al., 1984] or smoking history (cumulative pack-years [Livingston and Fineman, 1983]) is assessed. In contrast, cigarette smoking seems not to affect MN frequency [Bolognesi et al., 1993; Norppa et al., 1993; Stierum et al., 1993; Van Hummelen et al., 1993; Bonassi et al., 1994; Pitarque et al., 1996; Thierens et al., 1996], although at least one study reported a significant association between MN and cigarette smoking and alcohol consumption [da Cruz et al., 1994]. Reports on the possible effects of coffee and alcohol intake are scant and the conclusions largely discordant [Obe and Ristow, 1979; Obe et al., 1979, 1980; Reidy et al., 1988; Sarto et al., 1987; Nordic Study Group, 1990; Köteles et al., 1993; Nehlig and Derby, 1994]. The individual contribution of these habits cannot easily be assessed for linkage with smoking habits. However, there is plausible biological evidence suggesting that such drinking habits could have genetic effects in vivo: e.g., organic extracts from urine of heavy coffee drinkers were demonstrated to be mutagenic in *Salmonella typhimurium* [Dunn and Curtis, 1985; Nehling and Derby, 1994], while acetaldehyde, the first metabolic product of ethanol, induced SCEs both in vitro and in vivo [Obe and Ristow, 1979; Obe et al., 1979, 1980].

The availability of a large human population sample composed of 1,650 donors, selected from the general population, and well-characterized by a detailed questionnaire prompted us to investigate the possible effect of different biological and lifestyle factors on mean values of SCEs and MN frequencies. Sampling extended over 31 months. Data were analyzed taking into account several methodological factors known to affect the response of cytogenetic endpoints, in particular the period of sampling, which has been found to exert a strong modulating effect on overall mean SCE values [Tucker et al., 1987; Anderson et al., 1991; Barale et al., 1998]. Data, adjusted for this confounding variable, allowed more correct analysis of the above-mentioned biological and lifestyle factors. Furthermore, the large sample analyzed made it possible to study the rare individuals who declared themselves to be ''smokers only'' or ''coffee drinkers only'' or ''alcohol drinkers only.'' Therefore, the present data could shed some light on the intriguing question as to whether coffee and alcohol could be responsible, alone or interacting with smoking habits, for inducing SCEs and MN in humans.

MATERIALS AND METHODS

Donors

Selected subjects living in Pisa and in two nearby country towns, Cascina and Navacchio (Ca-Na), were contacted individually for participation in the study. Two thousand individuals accepted and, for 1,650 of these (83%), slides suitable for an adequate analysis of all cytogenetic endpoints were obtained. The three populations were sampled in two different periods: June 1991–November 1992 for Ca-Na and December 1992–November 1993 for Pisa. A detailed questionnaire was filled in by the volunteers under the supervision of specialized personnel. Further information including the demography of the population has been described elsewhere [Barale et al., 1998].

Lymphocyte Culture

Blood samples from 2,000 healthy donors were obtained by venipuncture, stored, processed, and cultured as previously described according to standard procedures. A heparinized whole-blood sample (0.3 mL) was added to 4.7 mL of culture medium composed of 4.025 mL Ham's F10 medium (ICN, Irvine, CA) supplemented with 0.5 mL (10%) fetal calf serum (ICN), 0.075 mL (1.5%) phytohemagglutinin (PHA, Wellcome, Pomezia, Italy) and antibiotics (100 IU penicillin and 100 μg/ml streptomycin; Sigma, St. Louis, MO).

SCE Analysis

5-Bromo-2′-deoxyuridine (BrdUrd; Sigma) was added to cultures (9 μg/mL) for the entire incubation period of 72 hr. Many investigators were involved both in culture processing and slide scoring. Investigators were rotated in order to homogenize differences in slide reading, while cultures were set up randomly in three different laboratories of the same department. The contribution of many experimental variables was assessed in a previous paper [Barale et al., 1998]. SCEs were evaluated by scoring 50 second metaphases with 46 chromosomes using five scorers (ten metaphases each). Proliferation indices (PIs) were also assessed according to the formula: PI = (MI + 2MII + 3MIII)/100.

MN Analysis

Cells were arrested to cytodieresis with cytochalasin B (3 μg/mL; Sigma) at 44 hr and harvested at 72 hr according to the CB-MN standard protocol. MN count was evaluated by scoring 1,000 binucleated cells/donor using five scorers (200 binucleated cells/scorer). The ratio binucleated cell/total cells (B/T) was also assessed as a possible indicator of cell proliferation after 72 hr of culturing.

Variables Considered

In the present study, age-, sex-, and lifestyle-related variables including smoking habits, coffee consumption, alcohol drinking, job, and site of residence were considered. Smoking habit was ranked as: 0 = never smoker; 1 = smoker, <10 cigarettes/day; 2 = smoker 10–19 cigarettes/day; 3 = smoker ≥20 cigarettes/day. Ex-smokers (383) were considered separately because their mean SCE values were found to depend on the duration of smoking cessation and MN means showed intermediate values (3.5‰ ± 0.23 vs. 3.25‰ ± 0.20 and 3.85‰ ± 0.19 of current smokers and never smokers, respectively). In addition, the average age of ex-smokers was significantly higher (more than 8 years) than that of other studied subjects and males were overrepresented (×2.2). The final sample, never smokers and current smokers, consisted of 1,250 donors. Coffee drinkers were ranked as: 0 = nondrinker; 1 = drinker ≤ 3 cups/day; 2 = drinker > 3 cups/day. Alcohol drinkers were ranked as: 0 = nondrinker, 1 = light drinker (≤0.5 liter/day of wine or beer), 2 = heavy drinker (>0.5 liter/day of wine or beer). The very few donors declaring themselves to be regular drinkers of high-proof spirits were grouped in level 2 as such. Alcoholics were excluded from the study. Job type was ranked as follows: 0 = student; 1 = pensioner; 2 = unemployed; 3 = housewife; 4 = white collar; 5 = blue collar. Blue-collar workers were mainly drivers, transport workers, or people working in farms or industries. White-collar workers were mainly teachers or people working in public offices, banks, or professional practices.

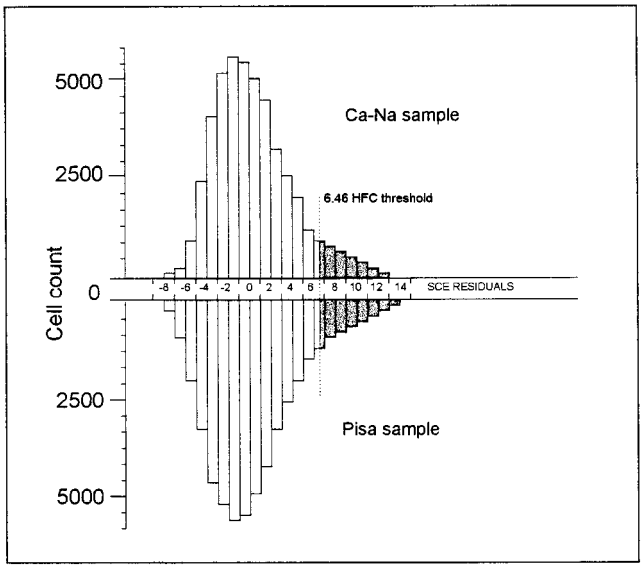


Fig. 1. Frequency histogram distribution of all the scored Sister Chromatid Exchanges (SCE counts) in the populations of Pisa and Ca-Na after adjusting for incubator and month factors (residuals). The 95th percentile is highlighted in order to account for the HFC threshold limit.

Statistical Analyses

Mean values of SCEs, PIs, and B/T ratios were distributed according to a Gaussian distribution (data not shown). MN counts were square-root transformed, allowing the best approach to a Gaussian. One-way analysis of variance (ANOVA), multifactor analysis of variance (MANOVA), and linear multiple regression analysis (MRA) were applied to study the effect of variables. When MRA was applied, categorical factors were considered as ''dummy'' variables. In addition, month of sampling (''month'') and incubator for lymphocyte culturing (''incubator'') factors were included in the model since they were previously demonstrated to strongly affect SCE and MN frequencies [Tucker et al., 1987; Anderson et al., 1991; Barale et al., 1998]. Since Ca-Na and Pisa donors sampling have been conducted in consecutive periods, month was considered as factor in MRA and MANOVA when the two populations were jointly analyzed. Similarly, high frequency cells (HFCs) were determined by replacing SCE counts with their ''residuals'' obtained by ANOVA considering month of sampling as a factor. Residuals were then processed as SCE counts to compute the threshold value of ''SCE residuals'' and to define ''HFC'' outliers with the confidence level of 95% [Carrano and Moore, 1982]. By this procedure, each SCE count, replaced by its residual, was adjusted for month fluctuation and other methodological variables, thus allowing comparisons among the subjects sampled throughout the study (Fig. 1).

The categorical variable (HFC outlier: yes/no) was further evaluated by means of multiple logistic regression analysis (MLR), taking into account sex, alcohol, coffee, smoking, job, and age as independent factors. In addition to SCE means, the dispersion of SCE counts/subject, i.e., the heterogeneity index (H = variance/mean), was also studied [Margolin and Shelby, 1985]. Since the variance is adjusted for the mean, H was expected to be less affected by month fluctuations than SCE means.

RESULTS AND DISCUSSION

Population Demography, SCEs, MN Frequencies, PI, and B/T Averages

In Table I a comprehensive description of the populations studied is given. Individuals were grouped according to site of residence, sex, and lifestyle factors, such as smoking, wine/beer, or coffee drinking. When age, sex ratio, job, and habits were considered, the two populations of Ca-Na and Pisa appeared almost homogeneous. In Table I average baseline frequencies of SCEs, PI, MN, and B/T, grouped differently according to the individually mentioned factors, are also given.

SCE and PI Analysis

Lifestyle: Alcohol, Coffee Consumption, and Smoking Habits

When donors were analyzed according to their lifestyle, it was observed that smokers consumed larger quantities of coffee and alcohol than nonsmokers, as previously observed in USA donors [Reidy et al., 1988; Hirsch et al., 1992]. For this reason, the assessment of possible correlations between cytogenetic endpoints, individual lifestyle factors, and their combination was difficult. Therefore, donors were preliminarily ranked in 12 classes, or ''habit ranks'' (HR), according to lifestyle factors (Table II). Moderate alcohol drinkers (less than 500 ml of wine or beer/day) were grouped with nondrinkers. In Figure 2, the frequency distribution of females and males according to HR clearly shows a trend for more intensive drinking and smoking among males than females.

Although some HR groups had only a few subjects, such as HR groups 5, 6, 7, and 8 (smokers, but not coffee drinkers), an evaluation of the net effect of smoking, coffee, and alcohol consumption was nevertheless attempted.

Preliminary MANOVA analyses (not shown) excluded two-factor interactions among HR, sex, job, and experimental factors, e.g., month of sampling and incubator used for cell culturing. The results of further MANOVA, without interactions, are shown in Table III. The HR variable removed a significant part of SCE variability explained by the model (14.6% in Ca-Na and 45.7% in Pisa). In order to identify the most important lifestyle factors, HR levels were analyzed considering level 1 as ''control'' (neither smokers nor coffee or wine drinkers) by MRA. In Table IV, the regression coefficients (± standard errors), sample size (n) and significance levels (p) are shown for mean values of SCEs.

Mean SCE values of donors falling into HR levels 10, 11, and 12 of Ca-Na and in HR levels 8, 10, and 12 of the Pisa population were significantly higher than in ''controls.'' However, the significance of the regression coefficient shown by level 11 in Pisa was statistically borderline, whereas results regarding level 8 introduced some uncertainties because of the limited sample sizes (n = 8 and n = 4 in Ca-Na and Pisa, respectively).

Although some groups were represented by few individuals, the present data seem to show that for the Ca-Na sample ''smokers only'' were effectively associated with increased mean SCE values. It can be seen from

TABLE I. Summary Statistics of Ca-Na and Pisa Populations

Ca-Na	Females			Males		
	n	SCEs	PI	n	SCEs	PI
Total	354	6.81 ± 1.35	2.31 ± 0.27	261	6.89 ± 1.36	2.33 ± 0.25
Smokers	79	7.13 ± 1.62	2.32 ± 0.25	121	7.44 ± 1.40	2.30 ± 0.26
Nonsmokers	275	6.72 ± 1.25	2.31 ± 0.27	140	6.42 ± 1.14	2.35 ± 0.24
Alcohol drinkers	143	6.93 ± 1.41	2.30 ± 0.29	137	6.92 ± 1.24	2.31 ± 0.24
Nondrinkers	209	6.73 ± 1.31	2.32 ± 0.25	101	6.67 ± 1.44	2.37 ± 0.25
Coffee drinkers	270	6.88 ± 1.38	2.31 ± 0.27	208	7.05 ± 1.39	2.33 ± 0.24
Nondrinkers	84	6.59 ± 1.23	2.32 ± 0.27	53	6.25 ± 1.06	2.35 ± 0.27
	n	B/T	MN	n	B/T	MN
Total	354	0.25 ± 0.16	4.65 ± 3.46	261	0.23 ± 0.15	3.91 ± 3.03
Smokers	79	0.27 ± 0.16	4.04 ± 3.27	121	0.23 ± 0.16	3.64 ± 2.68
Nonsmokers	275	0.24 ± 0.15	4.82 ± 3.50	140	0.23 ± 0.14	4.15 ± 3.29
Alcohol drinkers	143	0.25 ± 0.16	5.34 ± 3.52	137	0.24 ± 0.16	3.69 ± 2.78
Nondrinkers	209	0.25 ± 0.15	4.20 ± 3.36	101	0.22 ± 0.14	3.98 ± 3.26
Coffee drinkers	270	0.25 ± 0.16	4.67 ± 3.45	208	0.23 ± 0.15	4.00 ± 3.17
Nondrinkers	84	0.24 ± 0.15	4.59 ± 3.49	53	0.24 ± 0.16	3.56 ± 2.42
Pisa	Females			Males		
	n	SCEs	PI	n	SCEs	PI
Total	375	8.87 ± 1.62	2.39 ± 0.21	260	8.77 ± 1.65	2.41 ± 0.20
Smokers	99	9.62 ± 1.82	2.39 ± 0.18	128	9.35 ± 1.69	2.38 ± 0.20
Nonsmokers	276	8.60 ± 1.46	2.40 ± 0.21	132	8.21 ± 1.40	2.45 ± 0.19
Alcohol drinkers	142	8.83 ± 1.69	2.37 ± 0.21	135	8.82 ± 1.63	2.40 ± 0.19
Nondrinkers	229	8.86 ± 1.59	2.41 ± 0.20	102	8.48 ± 1.62	2.43 ± 0.20
Coffee drinkers	294	8.96 ± 1.63	2.39 ± 0.21	206	8.90 ± 1.60	2.41 ± 0.20
Nondrinkers	81	8.52 ± 1.55	2.41 ± 0.20	54	8.29 ± 1.76	2.43 ± 0.20
	n	B/T	MN	n	B/T	MN
Total	375	0.35 ± 0.11	3.57 ± 3.13	260	0.38 ± 0.12	2.68 ± 3.46
Smokers	99	0.34 ± 0.11	3.31 ± 2.90	128	0.39 ± 0.11	2.76 ± 4.18
Nonsmokers	276	0.35 ± 0.11	3.66 ± 3.21	132	0.37 ± 0.12	2.60 ± 2.60
Alcohol drinkers	142	0.33 ± 0.11	3.66 ± 3.00	135	0.37 ± 0.11	2.72 ± 2.60
Nondrinkers	229	0.36 ± 0.11	3.49 ± 3.23	102	0.38 ± 0.13	2.66 ± 4.60
Coffee drinkers	294	0.35 ± 0.11	3.58 ± 3.14	206	0.38 ± 0.11	2.83 ± 3.73
Nondrinkers	81	0.34 ± 0.11	3.53 ± 3.13	54	0.37 ± 0.13	2.09 ± 2.09

Table IV that mean SCE values of ''moderate smokers only'' (level 5, <20 cigarettes/day) was not significantly different from that observed in level 9 (moderate smokers + coffee drinkers) and in level 11 (moderate smokers + coffee + alcohol drinkers), which were both significantly higher than level 1. Therefore, among moderate smokers neither coffee nor alcohol drinking seemed to increase mean values of SCEs. Moreover, among heavy smokers no statistically significant differences have been found between ''heavy smokers only'' (level 6), ''smokers and coffee drinkers'' (level 10), and ''smokers, coffee and alcohol drinkers'' (level 12).

In sum, our data seemed to exclude interactions between smoking habits and coffee, wine, and beer drinking, but showed smoking habit as the only factor effectively associated with increased mean SCE values. It may be worth mentioning that the individuals in these groups exhibited a fairly sober smoking habit, since only 4.4% of subjects smoked >20 cigarettes/day and less then 0.9% of subjects smoked ≥ 40 cigarettes/day.

Since no interactions among lifestyle factors was observed, MRA was re-applied to the entire population by considering smoking, alcohol, and coffee drinking as independent factors. Table V shows the regression coefficients ± standard errors of MRA on SCEs.

The effect of smoking on mean values of SCEs appeared to be dose-dependent and detectable at the lowest smoking level tested (1–9 cigarettes/day). To our knowledge, it is the first time that an increase of SCEs is so clearly demonstrated even for very moderate smokers (≤ 9 cigarettes/day, average = 4.8 cigarettes/day). Since a different increase of SCEs in persons who smoked less or more than one pack/day has been reported [Soper et al., 1984], the possible dose-dependent effect of smoking was

TABLE II. Habit Rank (HR) According to Smoking and Coffee/Alcohol Drinking Habit (Sample Size in Brackets)

HR	Smoking level	Coffee cons.	Alcohol cons.	SCE means ± s.d. (n) Ca-Na	SCE means ± s.d. (n) Pisa
1	0	0	0 + 1	6.39 ± 1.15 (82)	8.31 ± 1.57 (98)
2	0	0	2	6.36 ± 1.15 (36)	8.18 ± 1.26 (28)
3	0	1 + 2	0 + 1	6.56 ± 1.21 (147)	8.63 ± 1.43 (146)
4	0	1 + 2	2	6.83 ± 1.26 (148)	8.45 ± 1.41 (141)
5	1 + 2	0	0 + 1	6.70 ± 1.00 (4)	8.21 ± 2.00 (6)
6	3	0	0 + 1	7.22 ± 0.98 (5)	9.45 ± 0.82 (3)
7	1 + 2	0	2	7.44 ± 1.66 (3)	10.64 ± — (1)
8	3	0	2	6.73 ± 1.46 (8)	11.46 ± 2.34 (4)
9	1 + 2	1 + 2	0 + 1	6.78 ± 1.24 (15)	8.88 ± 1.58 (26)
10	3	1 + 2	0 + 1	7.44 ± 1.73 (57)	9.64 ± 1.70 (57)
11	1 + 2	1 + 2	2	7.05 ± 1.23 (30)	9.06 ± 2.09 (36)
12	3	1 + 2	2	7.50 ± 1.48 (78)	9.64 ± 1.57 (96)

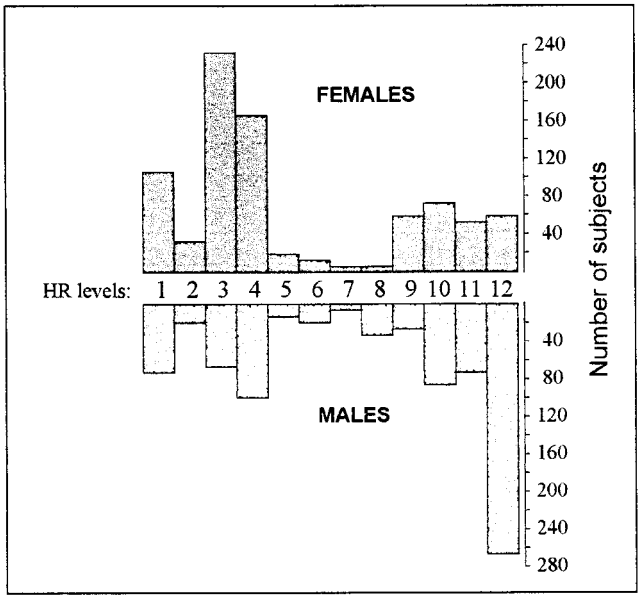


Fig. 2. Frequency histogram distribution of females (upper) and males (lower) grouped according to the HR variable (12 levels).

further investigated. Mean SCE values in smokers (n = 433) were regressed on the number of cigarettes/day (Fig. 3a). The linear regression coefficient (b = 0.060) was very close to the one (b = 0.054) obtained by Husum et al. [1986] in a study of 334 smokers. When analysis focused on very mild smokers (≤10 cigarettes/day, n = 195, Fig. 3b), the regression coefficient doubled (b = 0.098). In addition, the intercept value of this regression was 7.57, overlapping the mean of nonsmokers SCE value (7.53). Interestingly, taking into account age and methodological factors, smoking as few as 1–7 cigarettes/day (4.4 ± 1.5 cig/day) was still associated with a significant ($P = 0.018$) increase in mean SCE values (+5%) in females (n = 62) compared with nonsmoker females (8.1 ± 0.2 vs. 7.7 ± 0.06). The increase (+3%) in SCEs observed in males smoking an equivalent amount of cigarettes was not statistically significant (nonsmokers = 7.30 ± 0.7 vs. smokers = 7.55 ± 0.18; $P = 0.20$), probably due to the smaller number (n = 46) of males smoking so few cigarettes. By extending the number of cigarettes/day up to 10, and consequently the number of subjects (n = 92), a significant ($P = 0.001$) increase in SCEs was reached for males as well (nonsmokers = 7.30 ± 0.7 vs. smokers = 7.92 ± 0.13). For smoking females, MLR showed an increased SCE risk of 3% compared to males. This is exactly what one expects on the basis of the greater extension of chromosome target in females due to the presence of two X chromosomes (+3% of DNA).

Pack-years (average number of packs of cigarette/day multiplied per years of smoking) is considered a useful variable to describe smoking history. By considering age, sex, and methodological confounders, MRA did reveal highly significant ($P < 0.0001$) effect of pack-years (X) on mean SCE values: $Y = 5.70 + 0.024X$. This was partially expected, since pack-years was found to be strongly correlated with the number of cigarettes currently smoked/day ($Y = 8.53 + 0.31X$, $t_b = 14.4$, $P < 0.0001$, $r = .57$).

From MRA, coffee and alcohol drinking did not appear to be significantly associated with any modifications of SCEs (Table V) or with PI and H values (data not shown). A significant decrease of PI was also observed, but only in smokers >20 cigarettes/day, confirming the findings of Obe et al. [1982] and Reidy et al. [1988].

A borderline ($P = 0.055$) increase of SCEs (+0.28 ± 0.14) in heavy coffee drinkers was found (Table V). A significant effect of coffee consumption on SCEs has been already been reported elsewhere [Reidy et al., 1988; Hirsch et al., 1992]. The stronger effect found by these authors was probably due to the greater amount of coffee consumption observed in the donors enrolled in their study, whereas only 15% of coffee drinkers of the present study drank more than 3 cups/day, and less than 5% more

TABLE III. Sum of Squares, Degrees of Freedom, and Statistical Significance From Further MANOVA for Variables in the Order Fitted in MRA, Considering SCEs in Ca-Na and Pisa Populations

	Ca-Na		Pisa	
Factor	Sum of squares	d.f.	Sum of squares	d.f.
Month	162.80***	14	204.600***	11
Incubator	23.40***	1	0.065	1
Age	21.00***	1	15.240**	1
HR	37.96**	11	206.330***	11
Job type	9.14	5	14.060	5
Sex	4.79	1	11.230*	1
Model	259.08	33	451.500	30

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

TABLE IV. Regression Coefficient and Statistical Significance of Multiple Regression Analysis of SCE Frequency Observed in the Ca-Na and Pisa Population, on the Dummy Variable HR

	Ca-Na			Pisa			Pooled sample		
HR	Coeff. ± s.e.	*P*	n	Coeff. ± s.e.	*P*	n	Coeff. ± s.e.	*P*	n
2 vs. 1	0.07 ± 0.01	0.80	36	−0.23 ± 0.32	0.48	28	−0.160 ± 0.20	0.41	64
3 vs. 1	0.19 ± 0.20	0.35	147	0.10 ± 0.20	0.61	146	0.096 ± 0.13	0.46	293
4 vs. 1	0.18 ± 0.21	0.39	148	0.057 ± 0.21	0.79	141	0.111 ± 0.14	0.41	289
5 vs. 1	0.65 ± 0.83	0.43	4	0.051 ± 0.61	0.93	6	−0.014 ± 0.42	0.97	10
6 vs. 1	1.53 ± 0.82	0.06	5	0.877 ± 1.04	0.40	3	1.100 ± 0.50	0.03*	8
7 vs. 1	−0.48 ± 1.18	0.69	3	2.51 ± 1.46	0.09	1	1.230 ± 0.66	0.06	4
8 vs. 1	0.10 ± 0.68	0.88	8	2.66 ± 0.75	<0.001*	4	0.914 ± 0.39	0.02*	12
9 vs. 1	0.47 ± 0.38	0.21	15	0.39 ± 0.33	0.24	26	0.407 ± 0.23	0.07	41
10 vs. 1	0.93 ± 0.25	<0.001*	57	1.44 ± 0.26	<0.001*	57	1.160 ± 0.17	<0.001*	114
11 vs. 1	0.75 ± 0.30	0.015*	30	0.53 ± 0.30	0.08	36	0.560 ± 0.20	<0.001*	66
12 vs. 1	0.99 ± 0.25	<0.001*	78	1.32 ± 0.23	<0.001*	96	1.150 ± 0.15	<0.001*	174

than 5 cups/day. In addition, a cup of Italian coffee contains only 1/5 the water-soluble compounds, including caffeine, contained in Northern European and North American cups of coffee.

Analysis of HFC outliers by MLR is shown in Table VI. Results confirmed the outcome of analysis on mean values of SCEs. Smokers showed a statistically significant ($P < 0.001$) higher share of HFC outliers than nonsmokers. In addition, a statistically significant effect of coffee consumption on HFC outliers emerged in the total population (RR = 1.49, $P < 0.03$). The in vivo effects of coffee drinking have not been definitively assessed, but the present data further suggest a weak association between genotoxic effects and coffee consumption and an absence of interaction with other factors.

SCE means and HFC outliers are reported jointly in Table VII. The effect of smoking appeared to be detected more effectively by the HFC outlier method than by SCE means only in terms of percent increase over the control. However, further study of the distribution of SCEs among cells and of SCE means among subjects belonging to different HR classes has been undertaken, as suggested by others [Bender et al., 1992], and is currently in progress.

Ex-smokers

There were 383 ex-smokers eligible for complete statistical analysis. Mean age (51.65 ± 14) was significantly higher than never smokers (42.62 ± 62) and current smokers (43.81 ± 15). Ex-smokers showed mean values of SCEs (8.09 ± 1.88), intermediate between never smokers (7.54 ± 1.61) and current smokers (8.45 ± 1.94). MLR analysis revealed an increased relative risk of SCEs (RR = 1.03; CI = 1.01–1.06) in ex-smokers as compared to never smokers (RR = 1.00) and a decreased risk vs. current smokers (RR = 1.11; CI = 1.09–1.13). Ex-smokers showed a statistically significant ($P < 0.0001$) decrease in mean SCEs values as a function of duration of smoking cessation (0–30 years, Fig. 4). MRA indicated a borderline but significant reduction (−4%) in SCEs during the first 3 years of smoking cessation. This reduction doubled (−8%) within the following 5 years of smoking cessation. Further periods of no smoking resulted in a further, but less pronounced, reduction in relative risk (RR, Table VIII).

In previous studies, conflicting results have been obtained on the effect of smoking cessation. No significant

TABLE V. MRA Applied on Mean Values of SCEs (Residuals) in Lymphocytes of Donors of the Pooled Populations, After Adjusting for Age, ''Month,'' and ''Incubator''

Variables:	Coeff. ± Std. error	Sig. level
Smoking habit		
1–9 cig./day vs. Nonsmokers	0.410 ± 0.1264	0.0013**
10–19 cig./day vs. Nonsmokers	0.725 ± 0.1144	<0.001***
>19 cig./day vs. Nonsmokers	1.390 ± 0.1314	<0.001***
Coffee drinking		
≤3 cups/day vs. Nondrinkers	0.138 ± 0.0973	0.1567
>3 cups/day vs. Nondrinkers	0.276 ± 0.1436	0.0546
Wine or beer drinking		
≤500 ml/day vs. Nondrinkers	0.002 ± 0.0811	0.9843
>500 ml/day vs. Nondrinkers	0.299 ± 0.1976	0.1304
Job type		
Pensioners	0.218 ± 0.2000	0.2740
Unemployed vs. Students	0.410 ± 0.2157	0.0573
Housewives vs. Students	0.181 ± 0.1773	0.3070
White collar workers vs. Students	0.008 ± 0.1400	0.9551
Blue collar workers vs. Students	0.121 ± 0.1506	0.4224
Sex		
Males vs. Females	−0.225 ± 0.0857	0.0086**

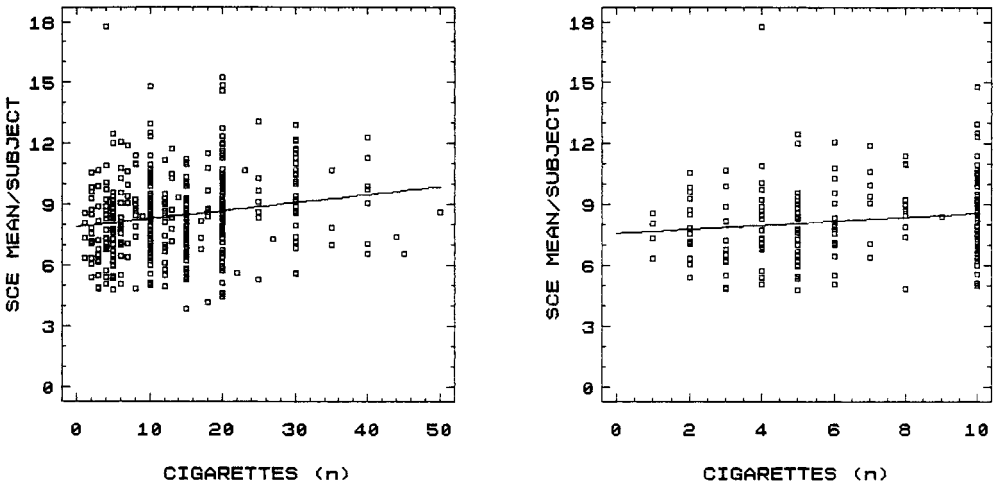


Fig. 3. (**left**) Regression analysis of SCE means/current smoker on the declared number of cigarettes smoked/day. Y = 7.88 + 0.04 cig/day (t_b = 3.86; $P < 0.001$). (**right**) As in (**left**) but smokers were selected for ≤10 cig/day (n = 194). Y = 7.57 + 0.98 cig/day (tb = 2.17; $P = 0.031$).

differences were observed between 7 ex-smokers and 24 never smokers by Livingston and Fineman [1983], nor by Hirsch et al. [1992], who compared 71 ex-smokers and 117 never smokers, or by Wulf et al. [1985] who performed a follow-up study for 124 days on 19 persons persuaded to stop smoking. In contrast, Sarto et al. [1987] found a significant decrease of SCEs in 14 ex-smokers monitored for 8 months after cessation. In the present study, based on the largest number of long-term ex-smokers (n = 383) so far investigated, the benefit of smoking cessation in decreasing mean values of SCEs was clearly demonstrated. Mean SCE values declined constantly during the first 8 years of smoking cessation, approaching the level of never smokers. These findings suggested prolonged persistence of irreparable DNA damage able to induce SCEs in long-lived lymphocytes of ex-smokers. Therefore, the apparent reduction in SCEs was more probably due to lymphocyte turnover rather than to DNA lesion removal. In this perspective, we have shown that at least one year of smoking cessation is necessary to ob-

TABLE VI. MLR Analysis of HFC Outlier Frequency and RR Assessment

	Ca-Na		Pisa		Pooled samples	
Factors	*P* Level	RR (CI)	*P* Level	RR (CI)	*P* Level	RR (CI)
Sex	0.027	0.40 (0.17–0.90)	0.004	0.45 (0.26–0.78)	<0.001	0.44 (0.28–0.68)
Alcohol drnking	0.366	1.32 (0.72–2.40)	0.675	1.09 (0.73–1.64)	0.340	1.17 (0.84–1.63)
Coffee drinking	0.097	1.76 (0.90–3.45)	0.472	1.18 (0.75–1.84)	0.030	1.49 (1.04–2.14)
Smoking habit	<0.001	2.19 (1.58–3.03)	<0.001	2.17 (1.73–2.72)	<0.001	2.12 (1.78–2.53)
Job type	0.228	0.87 (0.69–1.09)	0.131	1.12 (0.97–1.31)	0.850	0.99 (0.88–1.12)
Age	0.009	1.04 (1.01–1.06)	0.066	1.01 (1.00–1.03)	0.002	1.02 (1.00–1.03)

RR shows the relative risk for each category and CI the confidence interval. For sex, RR appears smaller than 1 because it refers to males vs. females.

TABLE VII. Dose-Dependent Effect of Smoking Habit (Cig./Day) and Smoking History (Pack/Years) on Mean Values of SCEs and Number of HFC Outliers in Pooled Populations

Smoking class cig./day (n. of donors)	Mean number of cig./day	SCE mean (±S.D.)	*P* (MANOVA)	Increase %	HFC outliers n. (%)	*P* (χ^2)	Increase %
0 (823)	0	7.53 ± 1.62	—	—	46 (5.5)	—	—
≤9 (120)	4.8	8.08 ± 1.92	<.001	7.3	21 (17.1)	<0.0001	211
10–19 (169)	12.6	8.32 ± 1.80	<.0001	10.4	30 (17.5)	<0.0001	218
≥20 (138)	25.6	8.94 ± 2.06	<.0001	18.6	46 (32.9)	<0.0001	498
Pack/years (n. of donors)	Mean number of pack/year	SCE mean (±S.D.)	*P* (MANOVA)	Increase %	HFC outliers n. (%)	*P* (χ^2)	Increase %
(823)	—	7.53 ± 1.62	—	—	46 (5.5)	—	—
≤7 (148)	3.7	8.10 ± 1.80	<.0001	7.6	18 (13.8)	<0.0001	150
8–23 (145)	12.9	8.52 ± 1.90	<.0001	13.1	36 (30.8)	<0.0001	460
≥24 (144)	36.2	8.81 ± 2.01	<.0001	17.0	43 (42.6)	<0.0001	674

serve a significant decrease of single DNA strand breaks in lymphocytes of ex-smokers [Frenzilli et al., 1997]. These findings are in agreement with the presence of two major subpopulations of lymphocytes having different mean lifespans (1 and 6 years, respectively) as recently assessed [Bogen, 1993].

Sex

Females averaged 5% more SCEs per cells than males in Pisa and 2.1% in Ca-Na (Fig. 5). When populations were pooled, the difference between females and males (+3%) was statistically highly significant ($P = 0.009$, Table V) and males showed a decreased relative risk of being HFC outliers of 0.44 (CI 0.28–0.68), as displayed in Table VI. These findings might be explained by the larger number of female chromosome targets (+3%). According to our findings, elevated SCE levels for females have been previously described [Hedner et al., 1982; Soper et al., 1984; Margolin and Shelby, 1985; Dewdney et al., 1986; Wulf et al., 1986; Anderson et al., 1986; Bender et al., 1988; Ho Park et al., 1992; Lazutka et al., 1994].

Job

Job type showed no significant associations with variations in SCEs, HFC, and PI. Within each class of job a very broad spectrum of activity and exposure were represented (Tables 3, 5, 6). Therefore, it is likely that some individuals effectively exposed to environmental genotoxicants were grouped with weakly exposed or unexposed subjects, thereby masking the statistical association between some degree of exposure and increase in SCEs. This point will be investigated in detail in the future.

Age

Cell proliferation rate was found to be negatively correlated with age, both in Pisa and in the Ca-Na population ($P < 0.05$; $b = -0.003 \pm 0.001$ and -0.004 ± 0.001, respectively). In Figure 5, the relationships between age (clustered in quartiles) and mean SCE values, classified according to sex and site of residence, are shown after adjusting for lifestyle factors. Aging appeared significantly associated with an increase in SCE means in Ca-Na and Pisa donors (Table III), with an average increase of 0.013 SCEs/cell/year.

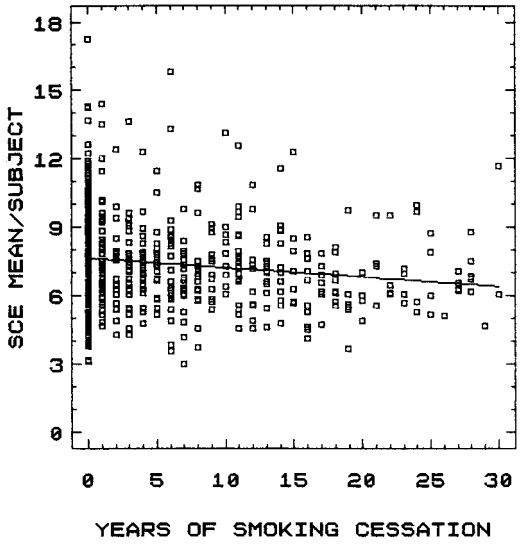


Fig. 4. Regression analysis of SCE means/ex-smokers on the years of smoking cessation corrected for age and other confounding factors. 0 years = current smokers. Y = 5.19−0.048 years of smoking cessation (tb = −5.7; $P < 0.001$).

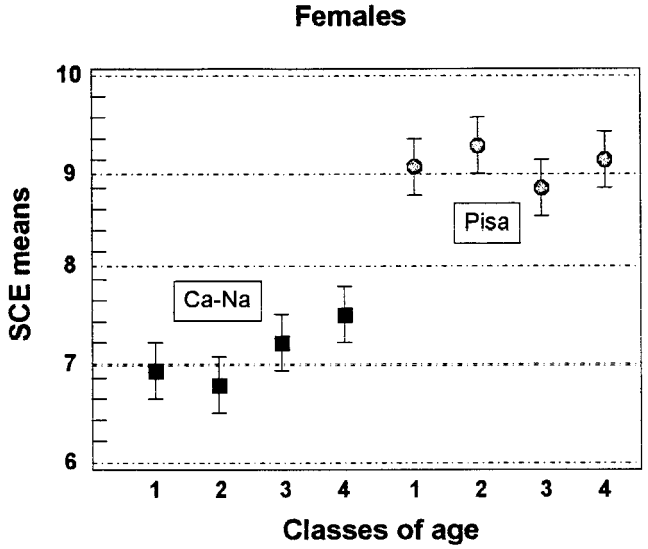
TABLE VIII. Smokng Cessation and Relative Risk (RR) Reduction of Mean Values of SCEs in Ex-smokers Compared to Current Smokers

Years of smoking cessation	(n)	RR	Reduction (%)	CI
0		1.00	—	—
0–3	(114)	0.96	4	0.93–0.99
4–8	(100)	0.92	8	0.88–0.96
9–17	(101)	0.90	10	0.87–0.94
>17	(68)	0.91	9	0.86–0.95

Share of Variance and Factors

The proportion of total variability explained by these variables was assessed by MRA in the two populations. Results are shown in piecharts (Fig. 6). The degree of variance generally unexplained by the models is impressive (67–75%). Some factors such as familiarity, diet, or specific professional exposure were not considered in the present study and could probably account for a fairly large portion of unexplained variability. It has been shown that genetic factors can account for about 30% of variation in mean SCE values [Hirsh et al., 1992].

Sex, age, habits, and job type accounted for a similar amount of variance both in Pisa and Ca-Na, although in the former group experimental variability was lower. In particular, age appeared to be one of the most important factors affecting PI (2.8% and 4.4% for the Ca-Na and Pisa population, respectively), whereas smoking habits represented a strong source of variability for SCE frequencies (5.5% and 12% of variance).



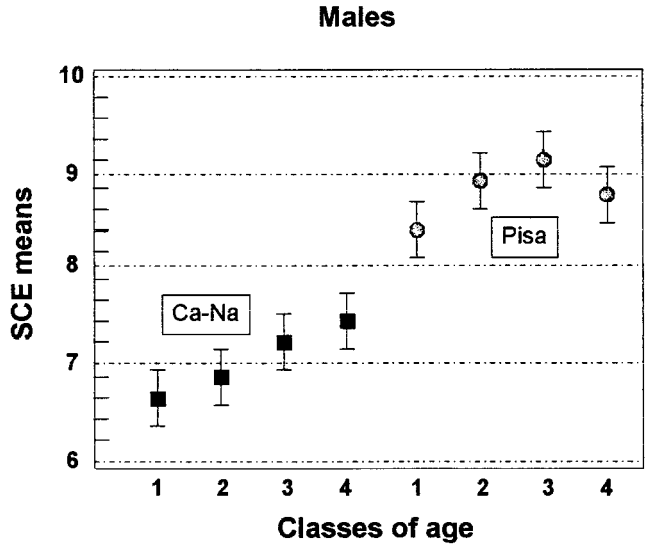


Fig. 5. Means and standard error bars of SCE means referring to males, females in Pisa, and Ca-Na samples, according to the different classes of age after adjusting data for the other confounding factors (smoking habit, coffee and alcohol consumption, job type). Class 1: <30 years; Class 2: 30–47 years; Class 3: 48–60 years; Class 4: >60 years.

H-index

As expected, H statistics showed smaller fluctuations through the months compared to mean values of SCEs, the variance being divided by the mean. This index was first introduced by Margolin and Shelby [1985] and then reproposed by Lazutka et al. [1994], supposing that H analysis could increase sensitivity of the assay. This analysis was regarded as similar and alternative to HFC evaluation. In the Pisa population, H differences among months were not statistically significant, and for the Ca-Na population the H-index removed less than 4.6% of total vari-

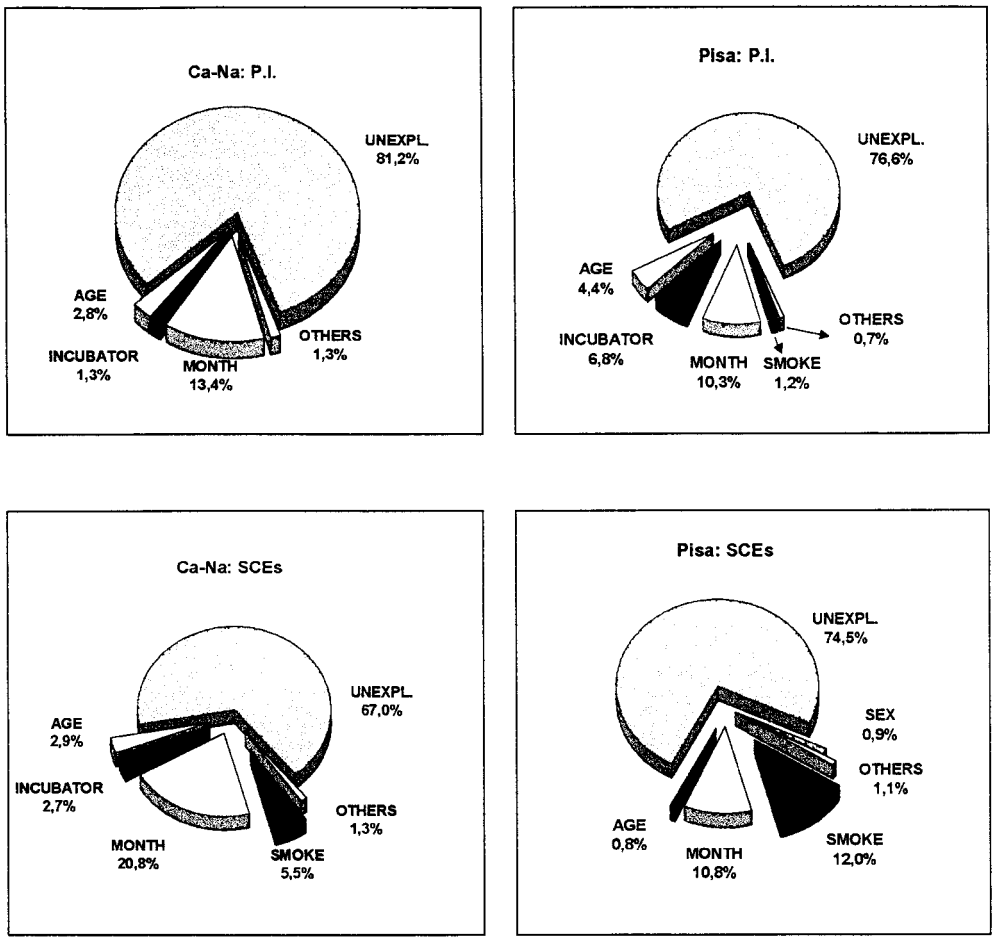


Fig. 6. Piechart showing the share of total variability attributable to each factor found to present a statistically significant association with SCE frequencies and PIs.

TABLE IX. Chi-Square Test for HFC Outlier Proportions Obtained in Pisa and Ca-Na Populations

	Pisa	Ca-Na	Totals
HFC outliers	104	39	143
Normal individuals	535	572	1,107
Totals	639	611	1,250
% HFC outliers	16.2%	6.4%	ratio: 2.5:1
Statistics	Chi-square: 37.8	D.F. = 1	P level < 0.001

ability, indicating it to be only slightly affected by month fluctuation. Unfortunately, the H-index proved to be less sensitive than SCE in regard to other factors, such as sex, age, and coffee and wine/beer drinking.

Differences Between Pisa and Ca-Na Populations

The different results obtained for Pisa and Ca-Na populations may be due to different genetic backgrounds or unknown environmental factors. The use of SCE count adjusted for experimental variability allowed a direct comparison of the two populations (Fig. 1). SCE residual distribution of Pisa showed a higher kurtosis value than Ca-Na (1.98 vs. 1.72). Consequently, a statistically significant higher share of HFC individuals was observed in the Pisa population by χ^2 test, as shown in Table IX. In spite of the fact that experimental variability was lower in the Pisa population, SCE distribution also showed higher standard deviation (3.83 vs. 3.22). This apparent discrepancy suggested that SCE counts of Pisa donors were affected by additional factors that increased sample variability, reducing the share of explained variance and determining a greater number of HFC outliers. Among younger people (former 50th percentile), HFC outliers were overrepresented in Pisa as compared to Ca-Na (3.5:1), whereas their ratio decreased (1.9:1) among older people. In particular, students showed an elevated Pisa/Ca-Na HFC outliers ratio (4.9:1), while pensioners showed no significant differences (1.4:1). On the contrary, blue-collar HFC outliers showed a very high Pisa/Ca-Na ratio (5.4:1), whereas white-collar HFC outliers of Pisa appeared slightly above (2.5:1) the general ratio (3.5:1).

TABLE X. Sum of Squares, Degrees of Freedom, and Statistical Significance (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) From Further MANOVA for Variables in the Order Fitted in MRA on MN Frequency in Ca-Na and PI Populations

	Ca-Na		PI	
	Sum of squares	d.f.	Sum of squares	d.f.
Month	0.0139*	14	0.0279***	11
Incubator	0.0270***	1	0.0663***	1
Age	0.0100***	1	0.0261***	1
HR	0.0110*	11	0.0070	11
Job activity	0.0020	5	0.0083*	5
Sex	0.0014	1	0.0064**	1
Model	0.0653	33	0.1420	30

TABLE XI. Regression Coefficient and Statistical Significance of Multiple Regression Analysis of $\sqrt{}$MN Frequency Observed in Ca-Na Population on the Dummy Variable HR

HR	Coeff. ± s.e.	*P*	n
2 vs. 1	0.0110 ± 0.0054	0.04*	36
3 vs. 1	−0.0018 ± 0.0039	0.64	147
4 vs. 1	0.0036 ± 0.0042	0.39	148
5 vs. 1	0.0039 ± 0.0162	0.81	4
6 vs. 1	−0.0107 ± 0.0161	0.50	5
7 vs. 1	0.0043 ± 0.0232	0.85	3
8 vs. 1	0.0013 ± 0.0133	0.92	8
9 vs. 1	−0.0045 ± 0.0074	0.54	15
10 vs. 1	0.0031 ± 0.0050	0.53	57
11 vs. 1	−0.0050 ± 0.0060	0.41	30
12 vs. 1	−0.0105 ± 0.0048	0.03*	78

Genetically, the Pisa population appeared more homogeneous than Ca-Na (ten polymorphic erythrocyte genes were studied [Mamolini et al., 1997]); therefore, the wide variability in individual cytogenetic response showed by Pisa people can not be attributed to higher genetic heterogeneity. These findings together suggested the presence of factors in the Pisa environment and/or population which increase the dispersion of data and particularly affect young people and blue collar workers. For example, the air quality monitoring system revealed a consistently higher pollution level in Pisa and preliminary results on donor health status revealed an increased frequency of allergies, respiratory symptoms, and illness in Pisa as compared to the Ca-Na populations [Viegi et al., 1991]. Correlation studies between cytogenetic endpoints and these indicators of health status have been initiated.

MN and B/T Ratios Analysis

Lifestyle: Alcohol Consumption, Coffee Drinking, and Smoking Habits

Preliminary MANOVA analyses (not shown) again excluded two-factor interactions for MN among HR, sex, job, and experimental factors such as month of sampling and incubator. Results from MANOVA, without interactions, are shown in Table X.

The HR variable was found statistically effective only in the Ca-Na population and its analysis by MRA is reported in Table XI. The most striking observation was the general negative slope of all the categories associated with smoking and coffee drinking (levels 9, 11, and 12), although statistical significance was reached only by level 12. The negative effect of smoking habits on MN frequencies was supported by the intermediate values of MN found in ex-smokers (data not shown). MRA was also carried out on pooled populations and smokers were negatively associated with MN frequency in a quantitative manner, whereas no significant effects were attributable to coffee or alcohol drinking (Table XII). In the same smokers, higher SCE frequencies has been herein reported, besides an increased chromosome aberration (CA) frequency [Milillo et al., 1996]. A similar disagreement between MN and CA analysis was observed by Obe et al. [1982]. Possibly a disturbance of lymphocyte proliferation rates in smokers may have generated ''false negative'' responses in the MN assay if damaged cells delayed their cell cycle. Previous studies failed to find any association between increased MN frequencies and smoking habits [Sorsa et al., 1988; Migliore et al., 1991; Bolognesi et al., 1993; Norppa et al., 1993; Stierum et al., 1993; Van Hummelen et al., 1993; Bonassi et al., 1994; Pitarque et al., 1996; Thierens et al., 1996]. On the other hand, some papers have reported a positive association [Hogstedt et al., 1983; Tomanin et al., 1991; da Cruz et al., 1994]. These data give the overall impression that the CB-MN assay is not as specific in detecting smoking effects as CA and SCEs. However, it has been shown that the subset of T8 lymphocytes are by far the most sensitive to smoking, since they showed a 15-fold difference in MN between smokers and nonsmokers [Larramendy and Knuutila, 1991]. Differential stimulation and/or proliferation of this particularly responsive subset of lymphocytes could be the cause of the controversial responsiveness.

A total lack of correlation ($P = 0.913$) between MN and SCEs of the whole population was found, further confirming the great difference underlying these two cytogenetic endpoints.

Age and Sex

The B/T ratio did not appear to be affected by aging (data not shown), although reduced cell cycling (PI) in older subjects has been shown. As previously observed [Fenech et al., 1986, 1994; Migliore et al., 1991], age was strongly associated in a positive way with MN frequency (Tables X, XII). Our data showed a significant increase

TABLE XII. MRA Applied on the Pooled Sample for MN Frequencies After Adjusting for the Experimental Variability

Variables	Coefficient ± std. error	Sig. level
Age	0.000335 ± 0.000069	<0.001***
Smoking: 1–9 cig./day vs. Nonsmokers	−0.007105 ± 0.002551	0.005**
Smoking: 10–19 cig/day vs. Nonsmokers	−0.005075 ± 0.002298	0.027*
Smoking: >19 cig./day vs. Nonsmokers	−0.004142 ± 0.002637	0.116
Coffee drinking: (≤3 cups/day): vs. Nondrinkers	−0.002694 ± 0.001947	0.166
Coffee drinking: (>3 cups/day) vs. Nondrinkers	0.000928 ± 0.002881	0.747
≤500 ml/day Wine or beer vs. Nondrinkers	0.000032 ± 0.001627	0.984
>500 ml/day Wine or beer vs. Nondrinkers	0.001117 ± 0.003976	0.778
Pensioners vs. Students	0.003146 ± 0.00403	0.435
Unemployed vs. Students	0.004935 ± 0.004382	0.260
Housewives vs. Students	0.003298 ± 0.00357	0.355
White collar workers vs. Students	0.007172 ± 0.002811	0.011
Blue collar workers vs. Students	0.007301 ± 0.003055	0.0017
Sex (F > M)	−0.005304 ± 0.00172	0.002**

TABLE XIII. MN Means (±s.d.) According to Sex and Decade Groups

Age groups	Females (n)	Males (n)	*P* value
≤19	2.20 ± 2.41 (61)	2.20 ± 2.04 (75)	0.8300
20 ≤ 29	2.79 ± 2.41 (121)	2.51 ± 2.61 (140)	0.2800
30 ≤ 39	3.50 ± 2.96 (100)	3.88 ± 4.97 (89)	0.4300
40 ≤ 49	4.47 ± 3.79 (174)	3.37 ± 2.76 (133)	0.0050
50 ≤ 59	4.69 ± 3.43 (168)	3.70 ± 3.21 (147)	0.0015
60 ≤ 69	4.22 ± 3.11 (161)	3.41 ± 3.05 (147)	0.0180
≥70	4.96 ± 4.02 (59)	3.57 ± 3.24 (57)	0.0280

Month sampling and ''incubator'' factors have been considered in MANOVA for determining the significance level (*P*) of observed differences between females and males. MN/1,000 binucleated cells.

of 0.032 MN/1000 binucleated lymphocytes/year. In the pooled populations (Table XII), sex was highly and significantly ($P = 0.002$) associated with MN frequencies, with higher values observed in females. The regression slope for males was 0.023 MN $\times 10^{-3}$ cells/year, while that for females was nearly double: 0.043 MN $\times 10^{-3}$ cells/year. This difference is statistically highly significant ($t = 5.4$, $P < 0.001$). Interestingly, the intercept with the Y axis was almost the same for both males and females: 2.014×10^{-3} MN vs. 2.184×10^{-3}($t = .011$, P = n.s.), suggesting that newborns should show the same MN frequency regardless of sex. However, by grouping donors into 10-year age periods, females showed statistically significant higher MN frequencies (+32%) only over 40 years of age (Table XIII). In addition, while in females MN frequency seemed to increase constantly with age, in males a plateau was reached at the age of 30–39 years. It is noteworthy that in a recent review the effect of sex was reported to determine an MN increase in females ranging between 21 and 38% [Bonassi et al., 1995]. The sex effect could be explained by the supposed preferential aneuploidogenic events involving the X-chromosome [Fenech et al., 1994; Bonassi et al., 1995]. In particular, in cultured lymphocytes the X chromosome has been found to be present from 72% [Hando et al., 1994] to 79.8% [Surrales et al., 1996] in MN obtained from cytochalsin B-induced binucleated cells; this is almost 18 times higher than expected.

Job Type

Job type showed a weak, but statistically significant, association with MN variations in Pisa, but not in the Ca-Na sample. In particular, white- and blue-collar workers showed, respectively, a statistically significant increase of 0.71‰ and 0.55‰ in MN frequency as compared to students after adjusting for age. This finding was not confirmed by analysis on the Ca-Na sample. To explain the observed discrepancy between the two sites, aneuploidogenic exposure related to job environment in the Pisa area could be suggested. However, when the two populations were pooled (Table XII), white- and blue-collar workers maintained a statistically higher MN frequency compared to students. A detailed analysis, entirely devoted to assessment of the possible sources of mutagen exposure of workers in the Pisa and Ca-Na populations, will be carried out in the near future.

Share of Variance Explained by the Factors

MRA allowed assessment of the extent of total variability explained by the variables considered, as shown by

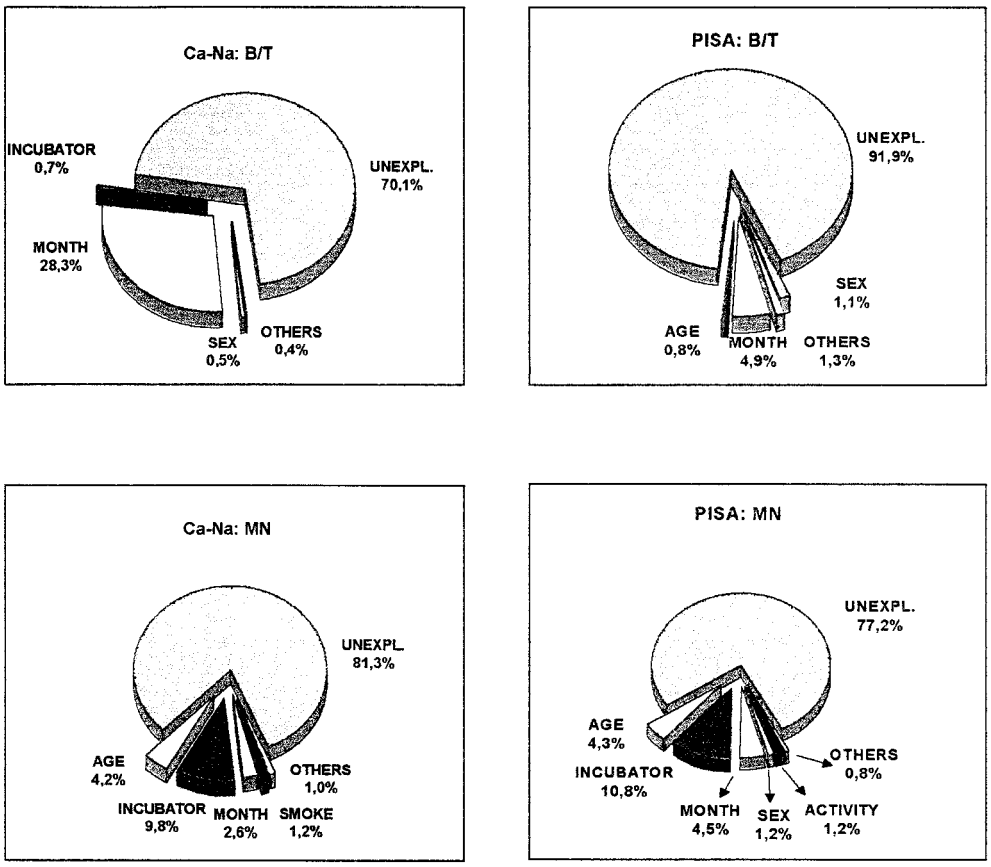


Fig. 7. Piechart showing the share of total variability attributable to each factor found to present a statistically significant association with MN frequencies and B/T.

the piecharts in Figure 7. In Figure 7, the contribution of several factors to B/T ratio variations in the Ca-Na and Pisa samples is shown. ''Month'' of sampling seemed to play a far larger role in the Ca-Na samples than in Pisa, while the ''incubator'' factor played a marginal or null role. Biological and lifestyle factors seemed to play a negligible role as well. Figure 7 shows the contribution of the same factors to percent MN variability. At first glance, all the most influential factors seemed to exert similar proportional effects in the two populations: age: 4.2% vs. 4.3%; incubator: 9.8% vs. 10.8%; month of sampling: 2.6% vs. 4.5%, respectively. Consequently, MN frequencies were affected by these variables more similarly in the two populations than B/T ratios, suggesting that the latter variable is likely to be more susceptible to culturing variations than to donor biological and lifestyle factors. However, the amount of variance unexplained by the models was still considerable (77–81%). Some factors, such as familiarity, diet, health status, or specific professional exposure, were not considered in the present study. It should also be pointed out that month of sampling and cell culturing incubator jointly accounted for 12–15% of variability, a very important share if compared to the individual effect of age (4.2–4.3%), sex, or smoking habits (less than 2%). In spite of this, the MN assay appeared able to detect an increased MN frequency in Pisa blue- and white-collar workers as compared to students (controls), accounting for 1.2% of total variability.

The observed differences between Ca-Na and Pisa could be explained either by an interaction with environmental factors, such as the higher air pollution level found in Pisa, and/or by a different genetic make-up of the two populations [Mamolini et al., 1997]. Note that the association between frequencies of CA and air pollution levels in the same populations has recently been shown [Milillo et al., 1996].

CONCLUSIONS

In the present study, the suitability of SCEs and MN analysis for detecting the effect of some biological and lifestyle-related factors was confirmed on a large human population.

The modulating effect on SCE and MN frequencies elicited by age and sex was clearly demonstrated and

quantified. Smoking habit was found to be by far the most effective lifestyle factor affecting SCEs in a general population, with a dose–effect relationship. It is worth mentioning that a statistically significant increase in SCEs was revealed even in the class of very moderate smokers (1–9/cigarettes/day) and particularly in females (1–7 cigarettes/day). Smoking cessation resulted in a definite reduction of SCEs in 1–8 years. In addition, the possible effect of coffee drinking (>3 cups/day) was suggested by the result of SCE means analysis and confirmed by the evaluation of HFC outliers. On the other hand, job effect was revealed only by MN assay, which, conversely, appeared not to be responsive to smoking habit. Nevertheless, when cytochalasin B free methods have been employed, the MN assay was effective in revealing the smoking habit [Hogstedt et al., 1983; Ganguly, 1993]. Interestingly, similar positive correlations have been obtained with the CB-MN assay when, compared to the standard protocol, lymphocyte incubation time was reduced up to 66 hr and the addition of cythochalasin B was brought forward to 42 hr [Tomanin et al., 1991]. The use of in situ hybridization of MNs with chromosome-specific centromeric probes in addition to further improvements in current CB-MN protocols will probably increase the reliability of cytochalasin B-MN assay in cytogenetic surveillance.

The observed differences between the two populations, or residence locations, remained largely unexplained, but further analysis on the correlation between SCEs, MN frequencies and health status may shed light upon these findings. Nevertheless, the present database will be of particular interest in the near future, when the follow-up of the present population will consider possible correlations between the cytogenetic endpoints investigated and the causes of morbidity and mortality of donors.

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REFERENCES

Anderson DA, Dewdney RS, Jenkinson PC, Lovell DR, Butterworth DP, Conning DM (1986): Sister chromatid exchanges (SCE) analysis in 106 control individuals. In Sorsa M, Norppa H (eds): ''Monitoring of Occupational Genotoxicants.'' New York: Alan R. Liss, pp 39–58.

Anderson D, Francis AJ, Godbert P, Jenkinson PC, Butterworth KR (1991): Chromosome aberrations (CA), sister-chromatid exchanges (SCEs) and mitogen-induced blastogenesis in cultured peripheral lymphocytes from 48 control individuals sampled 8 times over 2 years. Mutat Res 250:467–476.

Barale R, Marrazzini A, Bacci E, Di Sibio A, Tessa A, Cocchi L, Scarcelli V, Lubrano V, Vassale C, Landi S (1998): Sister chromatid exchange and micronucleus frequency in human lymphocytes of 1,650 subjects in an Italian population. I. Contribution of methodological factors. Environ Mol Mutagen 31:218–227.

Bender MA, Preston RJ, Leonard RC, Pyatt BE, Gooch PC, Shelby MD (1988): Chromosomal aberration and sister-chromatid exchange frequencies in peripheral blood lymphocytes of a large human population sample. Mutat Res 204:421–433.

Bender MA, Preston JR, Leonard RC, Pyatt BE, Gooch PC (1989): Chromosomal aberration and sister-chromatid exchange frequencies in peripheral blood lymphocytes of a large human population sample. II. Extension of age range. Mutat Res 212:149–154.

Bender MA, Preston RJ, Leonard RC, Pyatt BE, Gooch PC (1992): On the distribution of spontaneous SCE in human peripheral blood lymphocytes. Mutat Res 281:227–232.

Bogen KT (1993): Reassessment of human peripheral T-lymphocytes lifespan deduced from cytogenetic and cytotoxic effects of radiation. Int J Radiat Biol 64:195–204.

Bolognesi C, Parrini M, Merlo F, Bonassi S (1993): Frequency of micronuclei in lymphocytes from a group of floriculturists exposed to pesticides. J Toxicol Environ Health 40:405–411.

Bonassi S, Ceppi M, Fontana V, Merlo F. (1993): Multiple regression analysis of cytogenetic human data. Mutat Res 313:69–80.

Bonassi S, Bolognesi C, Abbondandolo A, Barale R, Bigatti P, Camurri L, Dalpra L, De Ferrari M, Forni A, Lando C, Padovani P, Pasquini R, Stella M, Puntoni R (1995): Influence of sex on cytogenetic end points: Evidence from a large human sample and review of the literature. Cancer Epidemiol Biomarkers Prev 4:671–679.

Brown T, Fox DP, Robertson FW, Bullock I (1983): Non-random chromosome loss in PHA-stimulated lymphocytes from normal individuals. Mutat Res 122:403–406.

Carrano AV, Moore DH (1982): The rationale and methodology for quantifying sister chromatid exchange in humans. In Heddle JA (ed): ''Mutagenicity: New Horizons in Genetic Toxicology.'' New York: Academic Press, pp 267–304.

da Cruz AD, McArthur AG, Silva CC, Curado MP, Glickman BW (1994): Human micronucleus counts are correlated with age, smoking, and cesium-137 dose in the Goiania (Brazil) radiological accident. Mutat Res 313:57–68.

Das BC, Rani R, Mitra AB, Luthra UK (1985): Baseline frequency of sister-chromatid exchanges (SCEs) in newborn lymphocytes and its relationship to in vivo aging in humans. Mutat Res 144:85–88.

de Arce MA (1981): The effect of donor sex and age on the number of sister chromatid exchanges in human lymphocytes growing in vitro. Hum Genet 57:83–85.

Dewdney RS, Lovell DP, Jenkinson PC, Anderson D (1986): Variation in sister chromatid exchange among 106 members of the general U.K. population. Mutat Res 171:43–51.

Dunn BP, Curtis JR (1985): Clastogenic agents in the urine of coffee drinkers and cigarette smokers, Mutat Res 147:179–188.

Fenech M, Morley AA (1985): The effect of donor age on spontaneous and induced micronuclei. Mutat Res 148:99–105.

Fenech M, Morley AA (1986): Cytokinesis-block micronucleus method in human lymphocytes: Effect of in vivo aging and low dose x-irradiation. Mutat Res 161:193–198.

Fenech M, Neville S, Rinald J (1994): Sex is an important variable affecting spontaneous micronucleus frequency of normal men and women. Hum Genet 39:329–337.

Ford JH, Russel JA (1985): Differences in the error mechanisms affecting sex and autosomal chromosomes in women of different ages within reproductive age group. Am J Hum Genet 37:973–983.

Frenzilli G, Betti C, Davini T, Desideri M, Fornai E, Giannessi L, Maggiorelli F, Paoletti P, Barale R (1997): Evaluation of DNA damage in leukocytes of ex-smokers by single cell gel electrophoresis (SCGE). Mutat Res, 375:117–123.

Ganuly BB (1993): Cell division, chromosomal damage and micronucleus formation in peripheral lymphocytes of healthy donors: Related to donor's age. Mutat Res 295:135–148.

Hando JC, Nath J, Tucker JD (1994): Sex chromosomes, micronuclei and aging in women. Chromosoma 103:186–192.

Hedner K, Hogstedt B, Kolnig AM, Mark-Vendel E, Strombeck B, Mitelman F (1982): Sister chromatid exchanges and structural chromosome aberrations in relation to age and sex. Hum Genet 62:305–309.

Hirsch BA, Sentz KK, McGue M (1992): Genetic and environmental influences on baseline SCEs. Environ Mol Mutagen 20:2–11.

Ho Park E, Kim JY, Byun DH, Lee JY, Lee JS (1992): Baseline frequency of sister-chromatid exchanges in 142 persons of the general Korean population. Mutat Res 268:239–246.

Hogstedt B, Gullberg B, Hedner K, Kolnig AM, Mitelman F, Skerving S, Widegren B (1983): Chromosome aberrations and micronuclei in bone marrow cells and peripheral blood lymphocytes in humans exposed to ethylene oxide. Hereditas 98:105–113.

Husum B, Wulf HC, Niebuhr E (1986): Sister chromatid exchange frequency correlates with age, sex and cigarette smoking in a 5-year material of 553 healthy adults. Hereditas 105:17–21.

Köteles GJ, Bojtor I, Szirmai S, Berces J, Otos M (1993): Micronucleus frequency in cultured lymphocytes of an urban population. Mutat Res 319:267–271.

Larramendy ML, Knuutila S (1991): Increased frequency of micronuclei in B- and T8-lymphocytes from smokers. Mutat Res 259:189–195.

Lazutka JR, Dedonyté V, Krapavickaitè D (1994): Sister-chromatid exchanges and their distribution in human lymphocytes in relation to age, sex and smoking. Mutat Res 306:173–180.

Livingston GK, Fineman RM (1983): Correlation of human lymphocyte SCE frequency with smoking history. Mutat Res 119:59–64.

Mamolini E, Beretta M, Barale R, Rodriguez-Larrelda A, Barrai I (1998): Detection of genetic structures at short distances in the Pisa area. Hum Hered, in press.

Margolin BH, Shelby MD (1985): Sister chromatid exchanges: A reexamination of the evidence for sex and race differences in humans. Environ Mol Mutagen 7(suppl 4):63–72.

Migliore L, Parrini M, Sbrana I, Biagini C, Battaglia A, Loprieno N (1991): Micronucleated lymphocytes in people occupationally exposed to potential environmental contaminants: The age effect. Mutat Res 256:13–20.

Milillo CP, Gemignani F, Sbrana I, Carrozzi L, Viegi G, Barale R (1996): Chromosome aberrations in humans in relation to site of residence. Mutat Res 360:173–179.

Morgan WF, Crossen PE (1977): The incidence of sister chromatid exchanges in cultured human lymphocytes. Mutat Res 42:305–312.

Nehlig A, Derby G (1994): Potential genotoxic, mutagenic and antimutagenic effects of coffee: A review. Mutat Res 317:145–162.

Nordic Study Goup on Health Risk of Chromosome Damage (1990): A Nordic data base on somatic chromosome damage in humans. Mutat Res 214:325–337.

Norppa H, Luomahaara S, Heikanen H, Roth S, Sorsa M, Renzi L, Lindholm C (1993): Micronucleus assay in lymphocytes as a tool to biomonitor human exposure to aneuploidogens and clastogens. Environ Health Perspect 101 (Suppl 3):139–143.

Nowinski GP, Van Dyke DL, Tilley BC, Jacobsen G, Babu VR, Worsham MJ, Wilson GN, Weiss L (1990): The frequency of aneuploidy in cultured lymphocytes is correlated with age and gender but not with reproductive history. Am J Hum Genet 46:1111.

Obe G, Ristow H (1979): Mutagenic, cancerogenic and teratogenic effects of alcohol. Mutat Res 65:229–259.

Obe G, Natarajan AT, Meyers M, Den Hertog A (1979): Induction of chromosomal aberrations in peripheral lymphocytes of human blood in vitro and of SCEs in bone-marrow cells of mice in vivo by ethanol and its metabolite acetaldehyde. Mutat Res 68:291–294.

Obe G, Gobel D, Engeln H, Herha J, Natarajan AT (1980): Chromosomal aberrations in peripheral lymphocytes of alcoholics. Mutat Res 73:377–386.

Obe G, Vogt H-J, Madle S, Fahning A, Heller WD (1982): Double blind study on the effect of cigarette smoking on the chromosomes of human peripheral blood lymphocytes in vivo. Mutat Res 92:309–345.

Pitarque M, Carbonell E, Lapena N, Marsa M, Torres M, Creus A, Xamena N, Marcos R (1996): No increase in micronuclei frequency in cultured blood lymphocytes from a group of filling station attendants. Mutat Res 367:161–167.

Reidy JA, Chen ATL, Welty TK (1988): Increased sister chromatid exchange associated with smoking and coffee consumption. Environ Mol Mutagen 12:311–318.

Richard F, Aurias A, Couturier J, Dutrillaux AM, Flury-Herard A, Gebault-Seureau M (1993): Aneuploidy in human lymphocytes: An extensive study of eight individuals of various ages. Mutat Res 295:71–80.

Sarto F, Faccioli MC, Cominato I, Levis AG (1985): Aging and smoking increase the frequency of sister-chromatid exchanges (SCEs) in man. Mutat Res 144:183–187.

Sarto F, Mustari L Mazzotti D, Tomatin R, Levis AG (1987): Variations of SCE frequencies in perpheral lymphocytes of ex-smokers. Mutat Res 192:157–162.

Soper KA, Stolley PD, Galloway SM, Smith JG, Nichols WW, Wolman SR (1984): Sister-chromatid exchanges (SCEs) report on control subjects in a study of occupationally exposed workers. Mutat Res 129:77–88.

Sorsa M, Husgafvel-Pursiainen K (1988): Assessment of passive and transplacental exposure to tobacco smoke. IARC Sci Publ 89:129–132.

Stierum RH, Hageman GJ, Welle IJ, Albering HJ, Schreurs JG, Kleinjans JC (1993): Evaluation of exposure reducing measures on parameters of genetic risk in a population occupationally exposed to coal fly ash. Mutat Res 319:245–255.

Surrales J, Falck G, Norppa H (1996): In vivo cytogenetic damage revealed by FISH analysis of micronuclei in uncultured human T lymphocytes. Cytogenet Cell Genet 75:151–154.

Thierens H, Vral A, De Ridder L (1996): A cytogenetic study of radiological workers: Effect of age, smoking and radiation burden on the micronucleus frequency. Mutat Res 360:75–82.

Tomanin R, Ballarin C, Nardini B, Mastrangelo G, Sarto F (1991): Influence of smoking habit on the frequency of micronuclei in human lymphocytes by the cytokinesis block method. Mutagenesis 6:123–126.

Tucker JD, Christensen ML, Strout CL, McGee KA, Carrano AV (1987): Variation in the human lymphocyte sister chromatid exchange frequency as a function of time: Results of daily and twice-weekly sampling. Environ Mol Mutagen 10:69–78.

Van Hummelen P, Gennart JP, Buchet JP, Lauwerys R, Kirsch-Volders M (1993): Biological markers in PAH exposed workers and controls. Mutat Res 300:231–239.

Viegi G, Paoletti P, Carrozzi L, Vellutini M, Diviggiano E, Di Pede C, Pistelli G, Giuntini G, Lebowitz MD (1991): Prevalence rates of respiratory symptoms in Italian general population samples exposed to different levels of air pollution. Environ Health Perspect 94:95–99.

Wulf HC, Husum B, Niebuhr E (1985): Cessation of smoking enhances sister chromatid exchanges in lymphocytes. Hereditas 102:195–198.

Wulf HC, Kousgaard N, Niebuhr E (1986): Sister chromatid exchange in childhood in relation to age and sex. Mutat Res 174:309–312.

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